High Throughput Protein, Protein-Protein Interaction, and Metabolite Assays CE

Claire Ouimet, Jing Nie, Erik Guetschow, Shuwen Sun & Shi Jin

Collaborators: David Lombard, Jason Gestwicki
High-speed Electrophoresis


- High E
- Short Distance
- Control Joule Heat (dimensions, conductivity)
- Narrow injection
- Sensitive detection

Applications of Fast Electrophoresis

- Western blot
- High Throughput Screening
- Non-covalent interactions
- “Sensing”
- 2-D Separations

* UNC Ramsey Group Website
Western Blotting: Most Used Affinity Separation

- Affinity and size information
- Ubiquitous (> 50,000 citations)
- Reliable
  - Slow, manual operation
  - Difficult for multiple proteins
  - Lack of Chip or CE equivalent

http://www.cellsignal.com/products/9946.html
Traditional Western Blot Workflow

Separate SDS-protein complexes (~1-3 hours)

Antibody specific for target

Immunoreaction (hours)

Block (30 min)

Target proteins

Detection

Blot (30-60 min)

Gel

Membrane

Filter paper

Blocking proteins

Target proteins
Recent Micro Western Developments

Fast Sieving Separations Possible in Chips with Entangled Polymer Media

Figure 5. Electropherogram of the separation of a BioRad protein ladder in optimized conditions (separation field 274 V/cm, and dilution ratio of 9).

Chip Based Western Blot

Separation field: 460 V/cm
Separation time: 2 min

Necessary to maintain stable current

Contains MeOH: Promote adsorption

Post-column Channel, 300 μm

Membrane

X-Y Translational Stage Motion

Syringe Pump

Deposited Proteins

S

11 mm

-1.4 kV

20 mm

SW

-1.8 kV

9 mm

BR
Resolution of ERK1/2 (42/44kDa) from Cell Lysate on Membrane

- On-column
- In sheath
- On membrane
Analysis of Multiple Proteins: “Stripping Analysis” in Conventional Western

• After 1st Western → remove antibody
• Apply 2nd Antibody
• Limited because of protein loss and time

www.piercenet.com
Multiple Separation Tracks on One Membrane (processed in parallel)

- Use repeated injection from 1 sample: multi-protein analysis
- Inject and deposit from multiple samples
- Process all immunoassays at 1 time
9 proteins, $n = 2$

800 nL sample
Microfluidic Western Blot Summary:
High Throughput, High Content

- Egg white Ladder
- INS-1

- Ladder
- 10 kDa
- 40 kDa
- 65 kDa

- Ladder
- Lysozyme
- AMPK
High Throughput Screening (HTS)

• 100,000 to 2,000,000 candidates for one target

• High density well plates / Robots / Optical Readout

Problems:
- Assay development
- False Signals
- Reagent use:
  Cost: $50k/screen
  (50 µL → 7.5 L for 150,000 samples)
  Difficult to express targets
Electrophoresis for Screening: Fast Assays

- Enzyme Assays
- Protein-Protein Interaction (Anal Chem 2013, 85:9824)
Fast Sample Introduction: Caliper High Throughput “Sipper Chip”

- Reads 384 Well Plate in 80 minutes
- Vacuum flow through channel is compromise
Segmented Flow for High Throughput Sample Delivery

Samples

Immiscible Fluid

Teflon tubing

During separation

+HV

Injection cross

Sampling channel

Separation channel

Waste channel

-HV

GND

Glass chip

PDMS chip

Extraction capillary
Droplet Extraction for Electrophoretic Analysis

Teflon Tube

Injection Cross

Extraction Capillary
Droplet MCE Screen: Sirt5 against Prestwick Library

- 384 samples
- 7 injections each
- 12 min
Comparison to LabChip System

Caliper LabChip (1 sample x 1 injection)

Droplet-CE (75 samples x 8 replicates = 600 injections)
Miniaturization of Screening

- Assays in 96, 384, and 1536 well plates
- Higher density have lower volume and use less reagent (saves costs for large scale screens)
- Going smaller than 1536 (1 uL) is difficult: evaporation, liquid handling
Miniaturization: All droplet assay
Protein–Protein Interaction as Drug Targets

- Long believed to be “undruggable”
- Large number of emerging targets
- Small molecule modulators identified recently

Part of human interactome

Protein-Protein Interaction Assays and Screens

Ideal: Fast, low sample consumption, label free, easy to adapt to new proteins, multi-protein complexes

- Surface Plasmon Resonance
- FRET
- Fluorescence Polarization
- Isothermal Calorimetry
- Bead & surface binding assays
Affinity Probe CE
(Noncompetitive Affinity Assay)

Free* Complex

Antibody-Antigen
Aptamer-target
Protein-peptide
Ligand-receptor
DNA-protein
Drug-apoenzyme

time
Chaperone Protein-Protein Interactions

- Hsp70-Bag
- Hsp90 dimer
- HOP
- substrate
- Hsp70
- Hsp90 dimer

- Chaperone interactions modulate client protein binding
- Proteostasis
- Cancer/Protein folding diseases

CE Assay for Protein-Protein Interaction

RFU

Time (s)

Hsp70* + Bag3

Hsp70* + Hsp70

Hsp70*
Current Standard Screen: Flow Cytometry Protein Interaction Assay

Streptavidin Bead

Biotinylated Hsp70

Streptavidin/Biotin linkage

AF488 label

Bead Associated Fluorescence Measured by Flow Cytometer

Bmax = 15879
Kd = 15.43

Comparison of CE and Bead Assay

3,443 Compounds Screened at 20 μM

CE

48 Hits (1.4%)

69% confirmed

100% reconfirm on FPCIA

Bead

118 Hits (3.4%)

Initial Screen

Dose-Response Curve

Cross Platform Test

50% confirmed

50% reconfirm on CE

Conclusion: CE is More Selective
Why is CE More Selective?

Bead Assay
- Polymer Bead
- AF488 label
- Bag3
- Hsp70

CE Assay
- Bag3
- Hsp70

Graphs:
- Normal compound
- Complex
- Free
- Aggregation agent
- Protein signal gone
- Particles
- Fluorescent compound
- Aggregation agent
- Normal compound

Time (min):
0 1 2 3
RFU:
0 30 60
Challenges for PPI Analysis by CE

Simultaneous: separations and interactions

Adsorption to Capillary

Complex dissociation

Protein Cross-linking Capillary Electrophoresis (PXCE)

gel electrophoresis

RFU

free complex

quench and denature

separate

time (min)
A high throughput dimer screening assay for monoclonal antibodies using chemical cross-linking and microchip electrophoresis.

Xiaoyu Chen¹, Gregory C. Flynn*  
Analytical and Formulation Sciences, Amgen Inc., Thousand Oaks, CA 91320, USA

Complexes Analyzed by PXCE

- **Free Hsp90**
- **Hsp90 Dimer**
- **2Hsp90-FKBP52**
- **Free Hsp70**
- **Hsp70-Bag3**
- **Free lysozyme**
- **Lysozyme-mab**
Effect of Hsp70-Bag3 Binding Site Mutants Detected by PXCE

Competitive Inhibition by PXCE: Hsp70-Bag3 mutants

- WT
- Hsp70 E,D 283,292 A,A
- Hsp70 R,R 258,262 A,A

Percent inhibition (%)

Log(Hsp70 conc.)

Log(K_i) by PXCE

Log(K_i) by FCPIA
Quantification of Small Molecule Inhibition by PXCE

Graph showing percent inhibition (%) vs. log(compound) for JG-311, JG-98, and JG-231. The graph also shows -log(IC50) for PXCE and FCPIA.

Legend:
- JG-311
- JG-98
- JG-231
- PXCE
- FCPIA
Fast CE for “Sensing”

- Aliquot Processing
- Sampling Probe
- Sample: Rxn, In vivo, Tissue
- Fast CE
- Time Resolved Chemical Information
“Sensing” Metabolites in the Living Brain:
Identify chemical signals in behavior, learning, pharmacology, pathophysiology

Challenges:
- Rapid chemical changes
- Spatially heterogeneous
- Delicate tissue
- >100 neurotransmitters + metabolites
- Freely moving animals
Microdialysis Sampling for In Vivo Monitoring

Microdialysis Probe

- Highly Versatile but...
- Low Temporal Resolution when coupled to HPLC because of large sample requirements (10 min)
Droplet Fraction Collection

On-line analysis

- collect seconds (nL) fractions
- analyze on-line or off-line
- minimal distortion due to flow
- CE compatible

Off-line Analysis

Collection Tubing
On-Board Droplet Generator for Awake Animal Experiments with Reagent Addition
Monitoring by Fast CE

Electrophoresis Trace

* Time of analysis, in vivo time = 20 s
Applications

- Effect of drugs on the brain
- Changes in transmitters with diseases
- Changes in transmitters with behavior
- Changes in transmitters with learning and memory
Huntington’s Disease

- “CAG Repeat” disease
- Neurodegenerative
- Cognitive and motor impairment
- Death within 15 years
- ~30k cases in USA
- Little treatment available
Glutamate Neurotransmission

Impaired glutamate uptake
Glu toxicity?
Recovery of Normal Glu Uptake in HD Mice with Cef
Ceftriaxone Improves Motor Performance

![Ceftriaxone Improves Motor Performance](image)

Graph showing clamping score over treatment days for R6/2 saline and R6/2 ceftriaxone groups. The graph indicates a significant improvement in motor performance for the ceftriaxone group compared to the saline group, as indicated by the asterisks denoting statistical significance.
Summary

- Fast, Multiplexed Western
- Droplet samples for high throughput
- Nanoliter lab
- PPI by CE gives high selectivity
- PXCE for troublesome proteins
- Fast CE for “sensing” in complex environments
Acknowledgements